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Hyun-Woo Lee April 18, 2012

Immunological and Virological Effects of PD-1 Blockade Post ART Interruption In the Blood of Chronically SIV Infected Indian Rhesus Macaques

by

Hyun-Woo Lee

Rama Rao Amara
Adviser

Department of Biology

Rama Rao Amara
Adviser

Arri Eisen

Committee Member

Patrick Cafferty

Committee Member

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Hyun-Woo Lee

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Abstract

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Chronic immunodeficiency virus infections are characterized by dysfunctional cellular antiviral immune responses. Virus-specific CD8 T cells progressively become exhausted and hierarchically lose functions such as abilities to proliferate and produce cytokines. Previous studies in various study models have shown that PD-1 inhibitory pathway could be targeted. In vivo PD-1 blockade in chronically simian immunodeficiency virus (SIV) infected rhesus macaques resulted in enhancement of immunological responses of SIV-specific CD8 T cells. Here we studied the immunological and virological effects of in vivo PD-1 blockade post antiretroviral therapy interruption (ART). Sixteen weeks after SIV infection Eleven Indian rhesus macaques received 21 weeks of ART. The administration of anti-PD-1 antibody was initiated four weeks after ART interruption. Mamu-A*01 tetramer was used to detect CD 8 T cells specific against immunodominant epitope Gag CM9 in the blood. The results show that PD-1 blockade post ART interruption resulted in a rapid decline in plasma viral load in four out of the seven anti-PD-1 antibody treated macaques. PD-1 blockade did not expand the frequency of Gag CM9-specific CD8 T cells, but increased the frequency of central memory-like CD8 T cells with high co-stimulatory and lymph node homing potential that could terminally differentiate into effector memory population to combat SIV infection. The findings suggest that the effects of PD-1 blockade may be dependent on maintenance of measurable levels of cytokine producing SIV-specific CD8 T cells following ART interruption.

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Purpose

The main objective of this study was to evaluate the immunological and virological effects of programmed death-1 (PD-1) blockade after antiretroviral therapy (ART) interruption. This was done by immunofluorescence of lymphocytes using whole blood samples from eleven chronically simian immunodeficiency virus (SIV) infected rhesus macaques.

Introduction

In 1983, a retrovirus called HIV was first isolated and identified as the causative agent of the acquired immunodeficiency syndrome (AIDS) (Hsiung, 1987). Since its identification almost three decades ago, AIDS has become one of the most devastating diseases in human history. According to the UNAIDS estimates, there are currently 34 million people living with HIV (UNAIDS, 2011). In the year 2010 alone, 2.7 million new cases of HIV infections and 1.8 million AIDS related deaths were reported (UNAIDS, 2011). Several antiretroviral drugs have been developed to fight HIV infection by interrupting various stages of the HIV replication cycle. Although ART is effective in suppressing plasma viral load, it cannot cure the infection since the establishment of a reservoir of latently infected cells allows the viral genome to persist (Han et al., 2007). Lifelong adherence to complicated, expensive and highly toxic drug regimen is suboptimal for the majority of people with HIV who live in low-income countries and lack access to stable medical care. Development of additional therapeutics is urgently needed.

HIV infection us comprised of various stages. Acute infection, which typically lasts about a week or two after viral infection, results in initiation of strong immune responses (McMichael

et al., 2010). Adaptive immune responses, particularly virus-specific CD8 T cell responses, are critical for the killing of intracellular pathogens. Virus-specific CD8 T cells recognize antigen by engaging the T cell receptor (TCR) with peptide-MHC I complex displayed on the surface of APCs (Anderson et al., 2006). Recognition of antigen stimulates T cell proliferation and differentiation into effector T cells (Anderson et al., 2006). Along with this dramatic proliferation, virus-specific CD8 T cells acquire antiviral functions, including the ability to produce cytokines, such as IFN-γ and TNF-α, to inhibit viral replication (Wherry & Ahmed, 2004). Initial robust immune responses result in a decrease in plasma viral load and an associated increase in CD4 T cell counts. If the infection is resolved, 90 to 95% of virus-specific CD8 cells undergo apoptosis, leaving behind a long-lived population of memory cells (Williams et al., 2008). These cells have an enhanced ability to control secondary exposure to antigen due to increased frequency, rapid acquisition of effector function, and less co-stimulation requirements (Kaech & Ahmed, 2011). However, the apparent control of HIV infection by the immune system is only transient. Latent HIV reservoirs are established during the acute phase of infection in cells located in the lymphatic tissue, leading to antigen persistence.

During the chronic phase of HIV infection, there is rapid, continuous, viral replication and a steady decline in CD4 T cell counts. Chronic infection is characterized by progressive impairment of effector T cell functions, and eventually physical deletion of virus-specific cells (Wherry et al., 2003). Exhaustion of virus-specific cells was first identified during persistent lymphocytic choriomeningitis (LCMV) infection of mice as effector immune cells that do not produce cytokines (Wherry, 2011). Exhausted virus-specific cells are less poly-functional compared with those seen during acute infections. It is thought that exhaustion evolved to apply some degree of immunological pressure on persisting pathogens, while preventing severe

immunopathology that would result if virus-specific cells that persist during chronic viral infections were as functionally responsive as memory CD8 T cells (Wherry, 2011). Loss of antiviral function occurs in a hierarchical manner, with exhausted virus-specific cells losing certain properties prior to losing others (See Fig. 1). In general, functions such as IL-2 production, proliferative capacity, and ex vivo killing are lost first. Other properties, such as the ability to produce TNF-α, are lost at more intermediate stage of dysfunction. Severe exhaustion eventually leads to virus-specific cells that partially or, in some cases, completely lack the ability to produce large amounts of IFN-γ. The final stage of exhaustion is physical deletion of virus-specific cells (Wherry, 2011). The severity of T cell exhaustion is correlated with increasing inhibitory receptor expression, high viral load, loss of CD4 T cell help, and prolonged infection (Wherry, 2011). Loss of T cell function greatly contributes to the inability of the immune system to clear invading pathogens. Exhaustion of virus-specific CD8 T cells in LCMV mice was quickly extended to other models including human immunodeficiency virus (HIV), SIV, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in humans (Velu et al., 2009).

Virus-specific CD8 T cells during chronic infection can co-express surface inhibitory receptors such as PD-1, LAG-3, TIM-3, CTLA-4, and many other inhibitory receptors (Wherry et al., 2011). PD-1 is a member of the extended CD28 family of T cell regulators. PD-1 is expressed on CD4 T cells, CD8 T cells, NK cells, B cells, and monocytes upon activation (Wherry et al., 2011). This broad expression contrasts with T cell-specific expression of CTLA-4, which suggests that PD-1 is involved in a broader spectrum of immune regulation than CTLA-4 (Parry et al. 2005). PD-1 has two ligands, PD-L1 and PD-L2, which are members of the B7 family. PD-L1 is expressed broadly on both professional and nonprofessional APCs and a wide variety of non-hematopoietic cell types (Freeman et al., 2006). PD-L2 expression is more

restricted and is expressed mainly by professional APCs such as dendritic cells (DCs) and macrophages. PD-1 ligand expression is up-regulated upon activation of antigen presenting cells (Freeman et al., 2006). Upon receptor engagement, two tyrosine signaling motifs on the cytoplasmic domain of PD-1 become phosphorylated. Phosphorylation of the second tyrosine, an immunoreceptor tyrosine-based switch motif, recruits the tyrosine phosphatases SHP-2 to PD-1 cytoplasmic domain. Recruitment of theses phosphatases leads to de-phosphorylation of TCR proximal signaling molecules, leading to attenuation of the TCR signal (Freeman et al., 2006). Analysis of the gene expression profile of exhausted virus-specific cells in chronic LCMV mice showed up-regulated PD-1 expression (Barber et al., 2006). In vivo administration of the anti-PD-L1 antibody expanded the frequency of LCMV-specific CD8 T cells and enhanced their functionality, restoring their ability to undergo proliferation, produce cytokines, and ex-vivo killing. Viremia was substantially reduced in the treated mice, and they were able to clear infection without exhibition of side effects (Barber et al., 2006).

The PD-1 pathway that was discovered in LCMV mice is conserved in humans and non-human primates. In vivo administration of the anti-PD-1 antibody in chronically SIV infected Indian rhesus macaques expanded the frequency of SIV-specific T cells with enhanced functionality in the blood and rectal mucosal tissues (Velu et al., 2009). PD-1 blockade was associated with reduction of plasma viral load, and higher rate of survival without any side effects (Velu et al., 2009).

Here we studied the immunological and virological effects of in vivo PD-1 blockade post ART interruption. The results showed that PD-1 blockade post ART interruption results in a rapid decline in plasma viral RNA levels in four out of the seven anti-PD-1 antibody treated macaques. PD-1 blockade post ART did not expand the frequency of Gag CM9-specific CD8 T

cells, but increased the frequency of central memory-like CD8 T cells with high co-stimulatory and lymph node homing potential that could terminally differentiate into effector memory population to combat SIV infection. The findings suggest that the effects of PD-1 blockade may be dependent on maintenance of measurable levels of cytokine producing SIV-specific CD8 T cells following ART interruption.

Materials and Methods

Study subjects. Two groups of Indian rhesus macaques were studied: Seven animals for anti-PD-1 antibody treatment and four animals to serve as controls. All macaques in this study were intra-rectally infected with a 10,000 tissue culture infectious dose (TCID50) of SIV251. Four out of seven animals in the PD-1 group, and two out of four animals from the control group expressed the Mamu-A*01 allele that express class I MHC complex capable of recognizing immunodominant epitope Gag CM9 (Sacha et al. 2007). All macaques were housed at the Yerkes National Primate Research Center and were cared for under the guidelines established by the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals," using protocols approved by the Emory University Institutional Animal Care and Use Committee.

Antiretroviral therapy. Sixteen weeks post SIV infection, all macaques were treated with ART for 21 weeks (See Fig. 2). The treatment regimen consisted of R-9-(2 phosphonylmethoxypropyl) adenine (PMPA) 20 mg/kg body weight, once daily subcutaneously, FTC (50 mg/kg, once daily subcutaneously), and Kaletra (Lopinavir 80 mg/kg, Ritonavir 20 mg/kg, twice daily orally). The treatment was interrupted at the end of 37 weeks post SIV infection.

Administration of anti-PD-1 antibody. Four weeks after ART interruption, seven animals in the PD-1 treatment group received anti-PD-1 antibody at a dose of 3mg/kg per animal (anti-human PD-1 antibody, clone EH-12) on days 0, 3, 7, 14, 21 and 28. Four in the control group received normal saline (See Fig. 2).

Analysis of the phenotypic expressions of Gag CM9-specific CD8 T cells. We followed the protocol for the staining we use regularly in our lab (Amara et al., 2001). Briefly, approximately 100μl of whole blood samples were surface-stained with FITC labeled anti-CD3 (SP34-2), Amcyan-labeled CD8 (Sk-1), PCP-labeled anti-CD4 (L-200), PECy7-labeled anti-CD28 (CD28.2), Percp-labeled anti-PD-1 (EH-12clone), PECy7-labeled anti-CD127 (A019D5), and Gag-CM9 (CTPYDINQM)-Mamu A*01 (Table 1). Following staining, samples were acquired using LSR II apparatus (BD Pharmingen, San Jose, California), and data were analyzed using FlowJo software (Treestar, Oregon, USA). (BD Pharmingen, San Jose, California).

Analysis of the cytokine production of SIV-specific CD8 T cells. We followed the protocol for the staining we use regularly in our lab (Amara et al., 2001). Briefly, approximately one million peripheral blood mononuclear cells (PBMC) were isolated from blood containing sodium citrate. PBMCs were stimulated for six hours with SIV Gag and Env peptides and incubated overnight at 4ºC. Cells were then stained with PB-labeled anti-CD3 (SP34-2), Amcyan-labeled CD8 (Sk-1), PCP-labeled anti-CD4 (L-200), PECy7-labeled anti-TNF- α (MAb11), PE-labeled anti-IL21 (4BG1), and A700-labeled anti-IFN- γ (B27) (Table 1). Samples were acquired using LSR II apparatus (BD Pharmingen, San Jose, California), and data were analyzed using FlowJo software (Treestar, Oregon, USA). (BD Pharmingen, San Jose, California), and PD-1 PE (Biolegend, San Diego, California).

Quantification of SIV copy numbers. The SIV copy number was determined using

quantitative real-time PCR. RNA was extracted from all specimens and amplified in duplicates, with the mean result reported.

Results

PD-1 blockade after ART interruption blunted re-emerging viremia. We monitored the temporal plasma viral RNA levels in the PD-1 treated and control group to evaluate the effects of PD-1 blockade post ART on viral control. Four out of seven macaques treated with anti-PD-1 antibody showed decline in viral RNA levels, while three showed no response. Unlike in the PD-1 treated group, viral load expanded in the controls. Based on the virological response that was observed, we divided the PD-1 treated macaques into two as responders and non-responders. Decline in viral load was seen in macaques from the responder group after PD-1 blockade post ART interruption, while no reduction was displayed by the non-responders and controls (See Fig. 3). In two animals from the responder group, viral load decreased 20-fold to 152.5 copies/ml (mean) by week 2 after the blockade. However, the levels rebounded to 11,045 copies/ml (mean) by week 8 after the blockade. The rebound in viral load in macaques corresponded with decreased rate of infused anti-PD-1 antibody and increased production of monkey antibody against anti-PD-1 antibody that took place during the period (data not shown). REe-12 from the responder group was able to continuously reduce viral load throughout the blockade from 5600 copies/ml to 118 copies/ml at week 4 after the blockade. Overall, the responder group was able to reduce the levels from 13767.5 copies/ml (mean) at week 0 to 5250.5 copies/ml (mean) at week 6 after the blockade. No significant viral load reduction was observed in the non-responders. The frequency of plasma viral RNA levels increased from

3916.67 copies/ml (mean) at week 0 to 7086.67 copies/ml (mean) at week 6 after the blockade. Exception of RKy-11, no viral control was observed in the control group. The frequency of viral load increased from 15137 copies/ml (mean) at week 4 to 79,433 copies/ml (mean) at week 8 after ART interruption. Long-term follow-up shows that the responders maintained lower viral set-point by week 24 following the blockade (See Fig. 4).

Responders had higher levels of IFN-γ + Gag-specific CD8 T cells at the initiation of PD-1 blockade post ART interruption. Previous studies have shown that IFN-γ produced by effector CD8 T cells is essential for direct inhibition of viral replication (Wherry & Ahmed, 2004). We monitored the temporal IFN-γ+ Gag CM9-specific CD8 T cells in the blood of the PD-1 treatment and control groups to evaluate the effect of PD-1 blockade post ART interruption on the frequency of IFN-γ+ SIV-specific CD8 T cells (See Fig. 5). At the initiation of the blockade, the responders had higher levels of IFN-γ+ SIV-specific CD8 T cells compared to the non-responders and controls. No expansion of IFN-γ+ SIV-specific CD8 T cells was observed in the responder group, while a small increase was displayed by the non-responders and controls. In the responders, the frequency decreased from 0.8648% (mean) at week 0 to 0.57% (mean) at week 10 after the blockade. In the low responders, the frequency increased from 0.0375% (mean) at week 0 to 0.05% (mean) at week 10 after the blockade. In the controls, the frequency increased from 0.14% (mean) at week 4 to 0.195% (mean) at week 10 after ART interruption. Similar results were obtained for TNF-alpha, IL-2, and IL-21 (data not shown).

Gag CM9-specific CD8 T cells declined following PD-1 blockade post ART interruption. We monitored the temporal Gag CM9-specific CD8 T cells in the blood of the PD-1 treatment and control groups to evaluate the effect of PD-1 blockade post ART interruption on

the frequency of SIV-specific CD8 T cells (See Fig. 6). At the initiation of the blockade, the responders had higher levels of virus-specific CD8 T cells compared to the non-responders and controls. No expansion of SIV-specific CD8 T cells was observed in the responders, while the frequency increased in the control animals. The frequency decreased from 5.45% (mean) at week 0 to 3.32% (mean) at week 10 after the blockade in the responders. The decline in SIV-specific CD8 T cells could be due to the decline in the viral load. In the control group, SIV-specific CD8 T cells expanded from 4.25% (mean) at week 4 to 5.99% (mean) at week 10 after ART interruption corresponding with increased plasma viral load.

Co-stimulatory molecule CD28 expression on Gag CM9-specific CD8 T cells increased in the responders after PD-1 blockade post ART interruption. CD28 is a co-stimulatory molecule that is highly expressed on naïve and central memory T cells, while the expression is minimal on effector memory population (Anderson et al., 2006). CD28 cross-links with B7, which provides amplification of TCR signaling for optimal activation and protection of CD8 T cells from anergy and cell induced death (Gibson et al. 1998). We studied the temporal CD28+ SIV-specific CD8 T cells in the blood of the responders and controls to monitor the percentage of central memory-like cells with co-stimulatory potential among SIV-specific CD8 T cells (See Fig. 7). The frequency of CD28+ Gag CM9-specific CD8 T cell transiently increased from 58.32% (mean) at week 0 to 63.27% (mean) at week 30 after the blockade in the responders. No expansion of CD28+ Gag CM9-specific CD8 T cells was observed throughout the trial in the controls. The frequency decreased from 69.192% (mean) at week 4 to 13.95% (mean) at week 34 after ART interruption.

CD127 expression on Gag CM9-specific CD8 T cells increased in the responders

after PD-1 blockade post ART interruption. CD127 binds with IL-7, an essential signaling molecule produced by CD4 T cells, DCs, and surrounding tissues for memory differentiation and homeostatic proliferation of memory cells (Anderson et al., 2006). Previous studies have shown that effector CD8 T cells down-regulate CD127 upon activation, but re-express it during differentiation in to memory T cells (Boettler et al. 2006). We studied the temporal CD127+ Gag CM9-specific CD8 T cells in the blood of the responders and controls in order to monitor the trend in SIV-specific effector CD8 T cells undergoing memory differentiation, and central memory CD8 T cells with proliferative potential after PD-1 blockade post ART interruption (See Fig. 8). The frequency of CD127+ Gag CM9-specific CD8 T cells increased in the responders, while no expansion was seen in the controls. Except REe-12, the frequency of CD127+ SIV-specific CD8 T cells in the responders increased from 16.1% (mean) at week 0 to 24.15% (mean) at week 4 post PD-1 blockade. Long-term follow-up shows that the responders up-regulated the frequency from 20.133% (mean) at week 4 to 33.533% (mean) at week 30 post PD-1 blockade. No expansion of CD127+ Gag CM9-specific CD8 T cell was observed in the controls. The frequency decreased from 12.4% (mean) at week 4 to 5.55% (mean) at week 34 post ART interruption.

PD-1 blockade enhanced the lymph node homing potential of Gag CM9-specific CD8 T cells in blood of the responders. Studies have shown that CCR7 codes for a chemokine receptor that plays a significant role in lymph node homing potential of naïve and central memory CD8 T cells (Geginat et al., 2003). We studied the temporal CCR7+ Gag CM9-specific CD8 T cells in order to monitor the effect of PD-1 blockade post ART interruption on the frequency of central memory-like cells with lymph homing potential among SIV-specific CD8 T cells (See Fig. 9). The frequency of CCR7+ SIV-specific CD8 T cells increased in the responders

from 7.47% (mean) at week 0 to 18.1% (mean) at week 30 after the blockade. No up-regulation of CCR7+ SIV-specific CD8 T cells was observed in the controls. The frequency decreased from 9.6% (mean) at week 4 to 6.14% (mean) at week 34 after ART interruption.

Discussion

More than 95% of all HIV infected individuals live in low-income countries that cannot provide stable access to antiretroviral drugs and permanent medical care (UNAIDS, 2011). Since access to ART is limited in such places, immunotherapy that could prime and enhance antiviral responses of one's own immune cells is the most effective way to combat HIV pandemic. Previous studies have shown that progressive dysfunction of virus-specific CD8 T cells occurs during chronic infection, and PD-1 blockade leads to reduction of plasma viral load, enhancement of the frequency and functionality of SIV-specific CD8 T cells without adverse side-effects.

Here we treated all macaques with ART prior to PD-1 blockade in order to encourage development of central memory cells that have lower activation requirements and more robust immune responses against antigens. In four out of seven animals, the treatment was effective at blunting initial plasma viral load levels, and increasing the percentage of central memory-like cells with lymphoid homing potential and co-stimulatory potential that could terminally differentiate into effector memory population to combat SIV infection. No expansion of cytokine producing SIV-specific was observed in the treatment group after the blockade. This is probably due to the fact that we started out with a relatively high frequency of Gag CM9-specific cytokine

producing CD8 T cells, unlike in the previous studies where CD8 T cells were highly dysfunctional to begin with. The controls that did not receive PD-1 blockade treatment after ART interruption failed to control viral load and showed progression of exhaustion of SIV-specific CD8 T cells indicated by rapid expansion of Gag CM-9 specific cells, but a minimal increase in the frequency of IFN-γ positive cells. In addition, low frequency of central memory-like cells with co-stimulatory and lymphoid homing potential was observed in the control group, indicating that effector virus-specific cells are unable to differentiate into memory population due to persistent antigen exposure.

Our results demonstrate that PD-1 blockade post ART interruption can significantly enhance viral control. However, the effects of blockade seem to be dependent on maintenance of measurable cytokine producing SIV-specific CD8 T cells following ART interruption. Immunological and virological effect of PD-1 blockade post ART interruption in rectal mucosal tissues and secondary lymphoid organs need to be studied in order to fully understand the effect of PD-1 blockade on viral control.

Table and Figures

Table 1. Fluorescent markers

Surface Staining Panel		**ICS Panel**
Panel 1	Panel 2	Panel
Ki67	CCR7	IL-21
CD4	P11C	CD4
P11C	CD127	IL-2
CD28	CD3	TNF-a
CD3	CD8	IFN-y
CD95	CD4	CD3
CD8		CD8

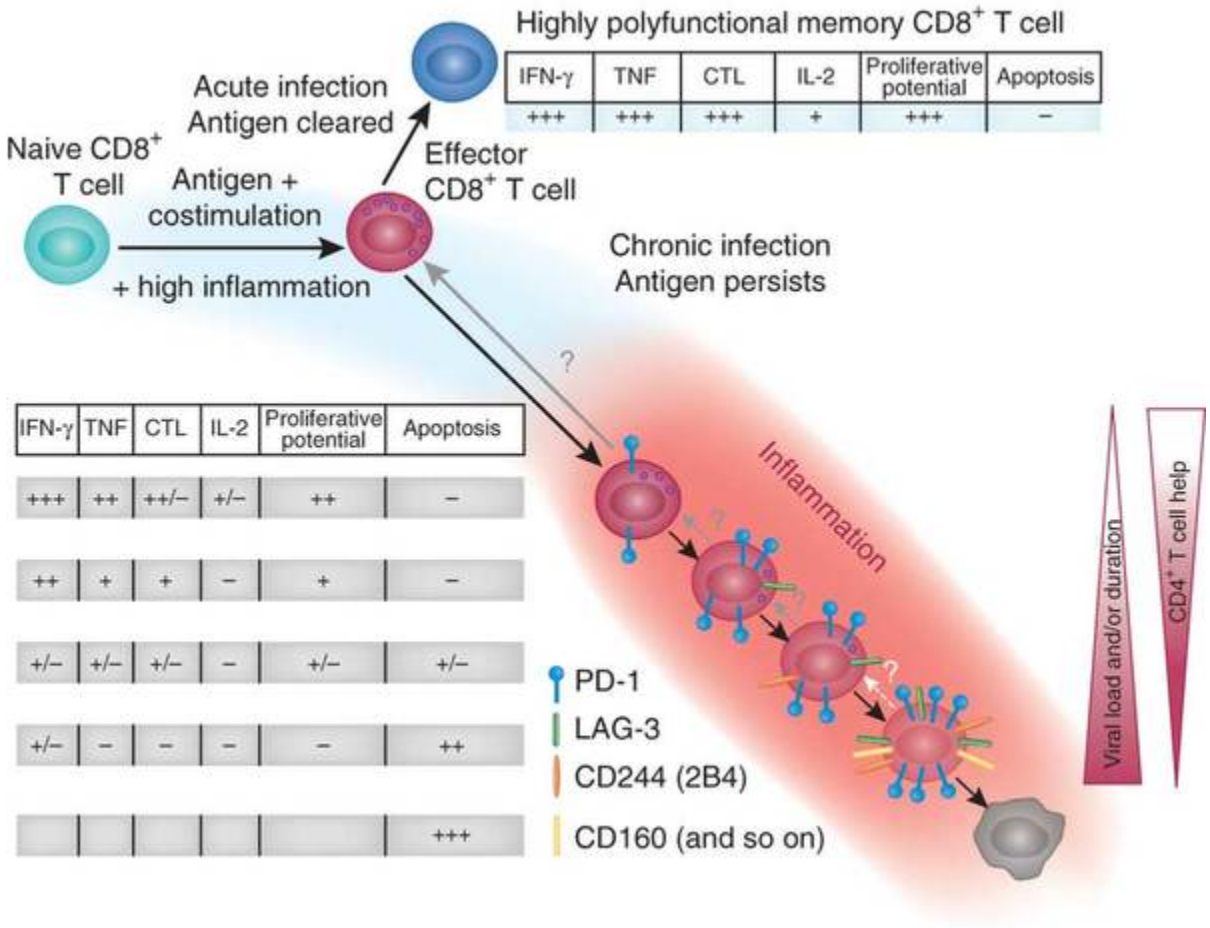

Fig. 1. Progressive dysfunction of pathogen-specific CD8 T cells. Loss of antiviral function occurs in a hierarchical manner, with exhausted cells losing certain properties prior to losing others. (Wherry, 2011)

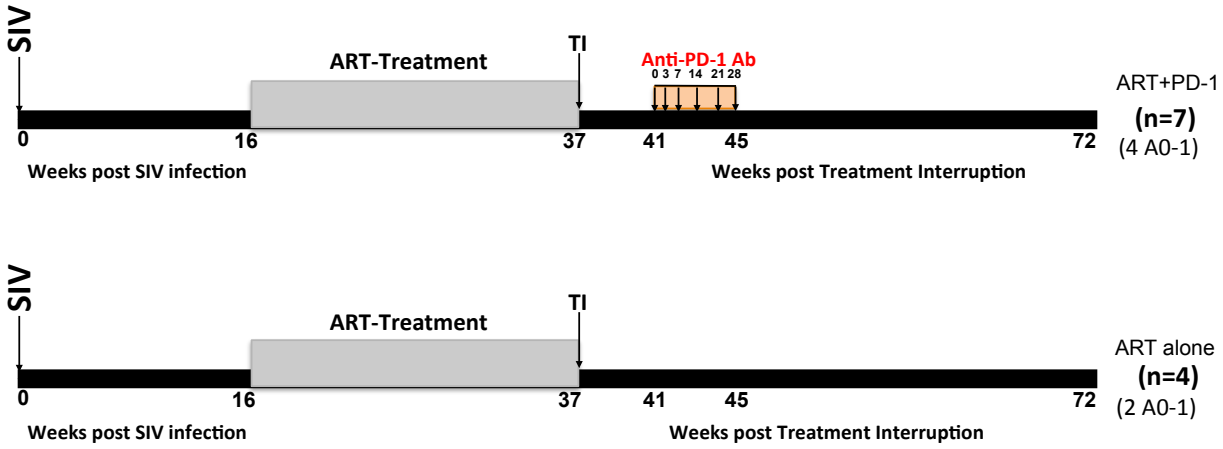

Fig. 2. Study design. All Indian rhesus macaques were treated with ART at wekk 16 for 21 weeks. Macaques in the treatment group received anti-PD-1 blockade after four weeks post ART interruption, while the controls received normal saline.

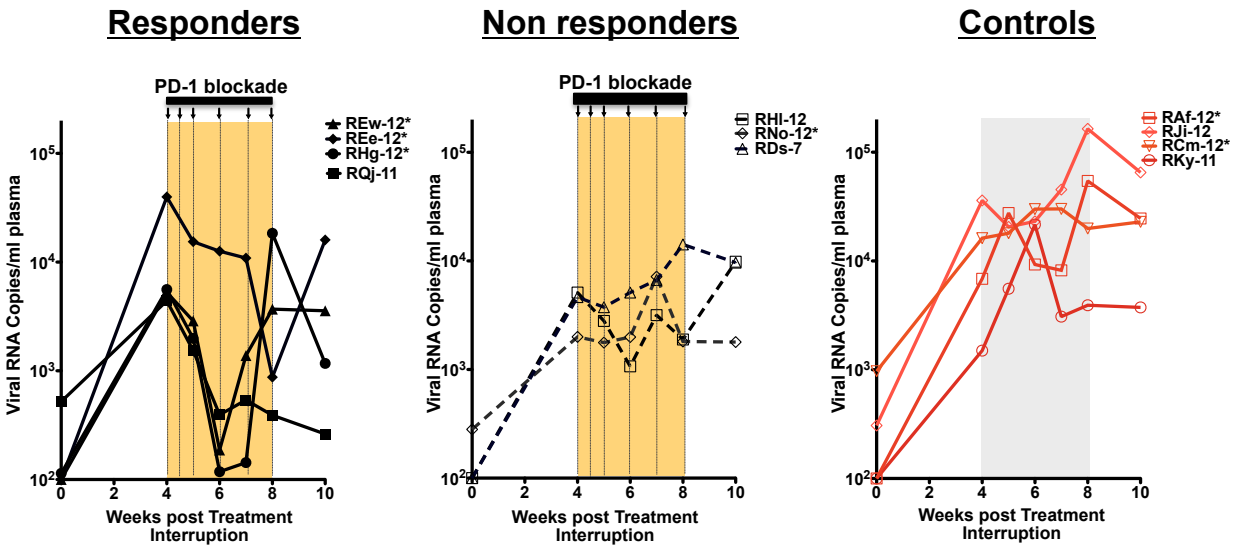

Fig. 3. Temporal plasma viral RNA copies/ml in the blood of different groups. Decline in viral load was seen in macaques from the responder group after PD-1 blockade post ART interruption.

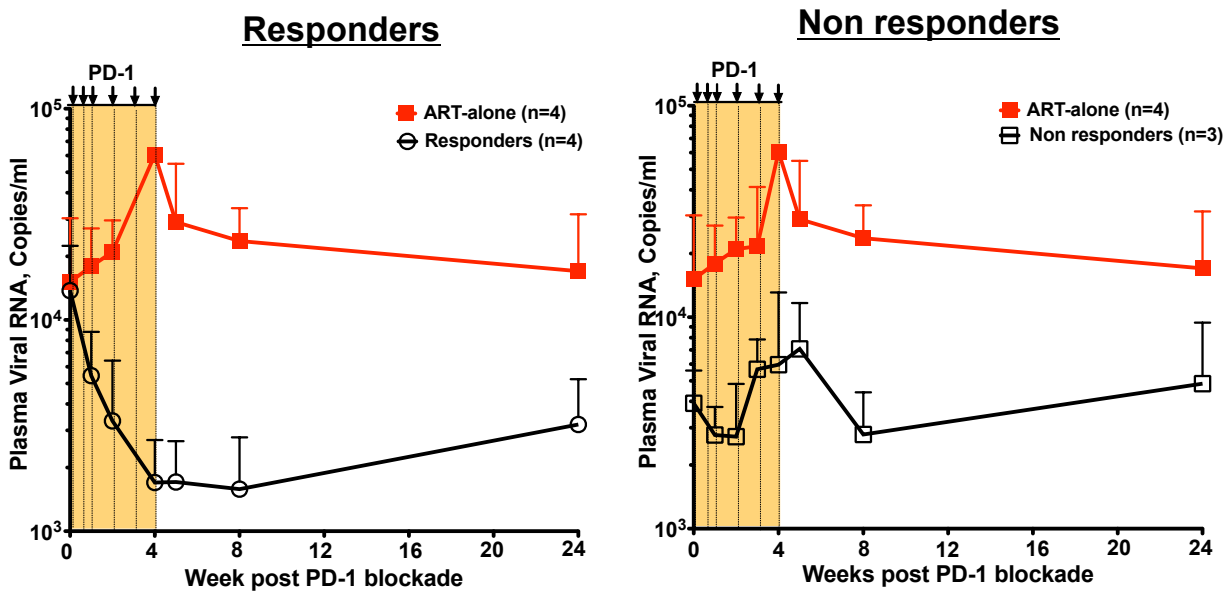

Fig. 4. Mean of temporal plasma viral RNA copies/ml in the blood of different groups after PD-blockade post ART interruption. The responders maintained lower viral set-point by week 24 following the blockade.

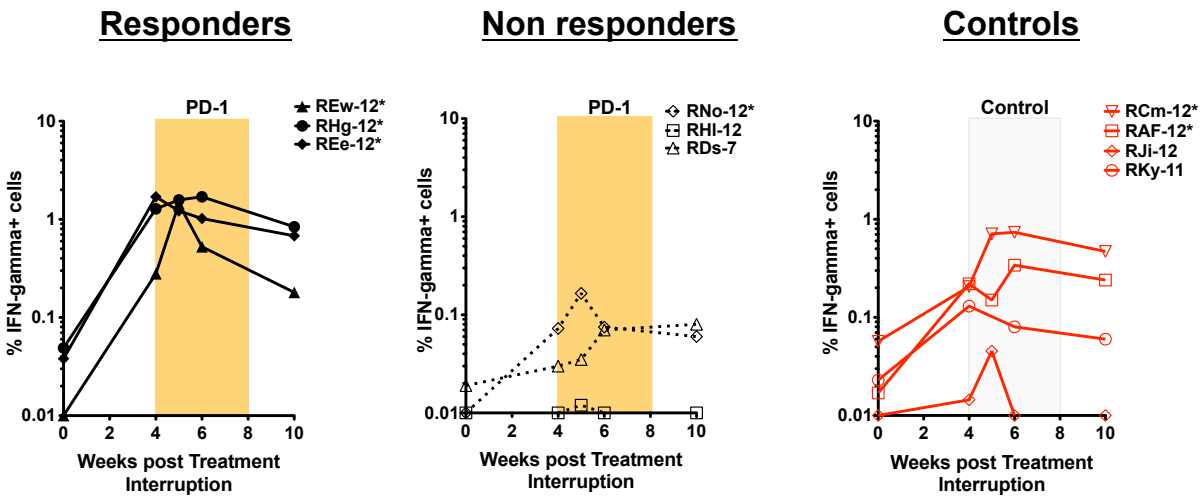

Fig. 5. Temporal IFN-γ+ Gag-specific CD8 T cells in the blood of different groups after PD-1 blockade post ART interruption. The responders started out with higher levels of cytokine-producing SIV-specific CD8 T cells.

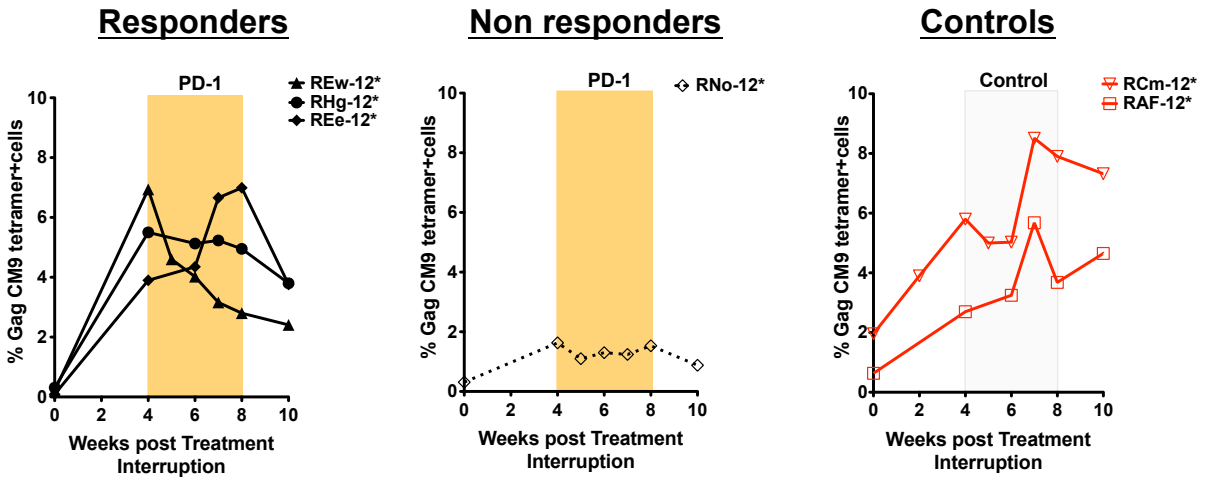

Fig. 6. Temporal Gag CM9-specific CD8 T cells in the blood of different groups after PD-1 blockade post interruption. At the initiation of the blockade, the responders had higher levels of virus-specific CD8 T cells compared to the non-responders and controls.

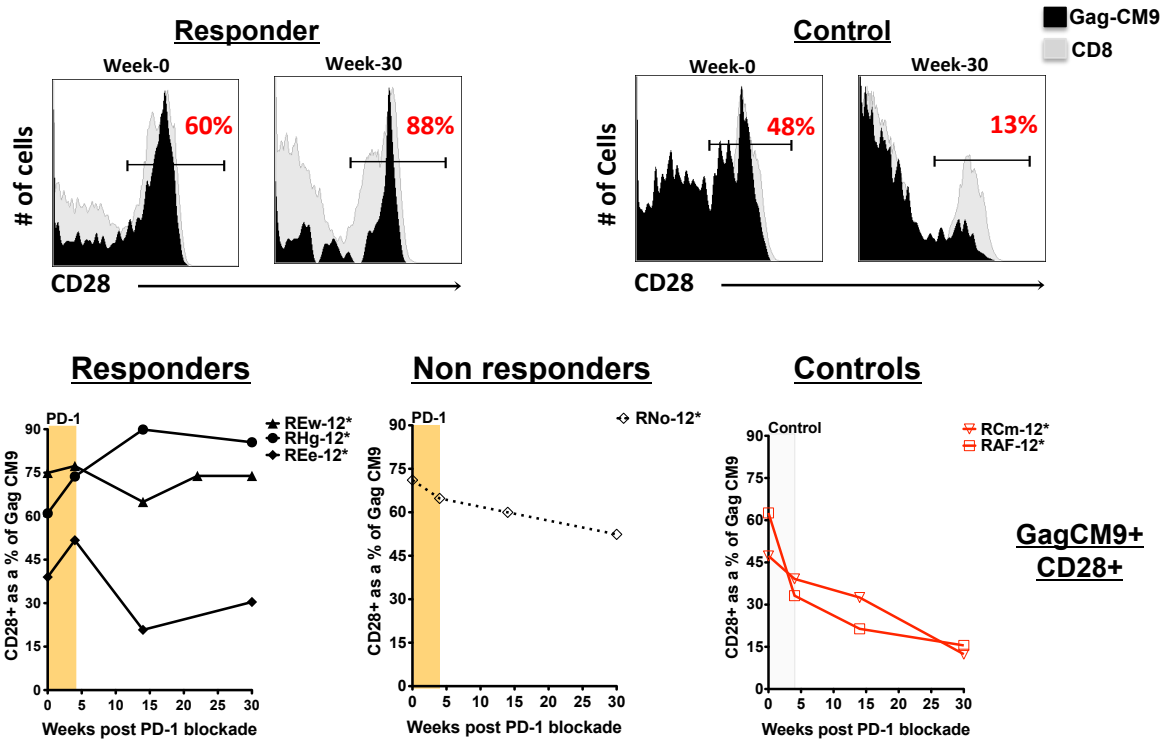

Fig. 7. Temporal CD28+ Gag CM9-specific CD8 T cells in the blood of different groups after PD-1 blockade post ART interruption. The frequency of CD28+ SIV-specific CD8 T cells increased in the responders.

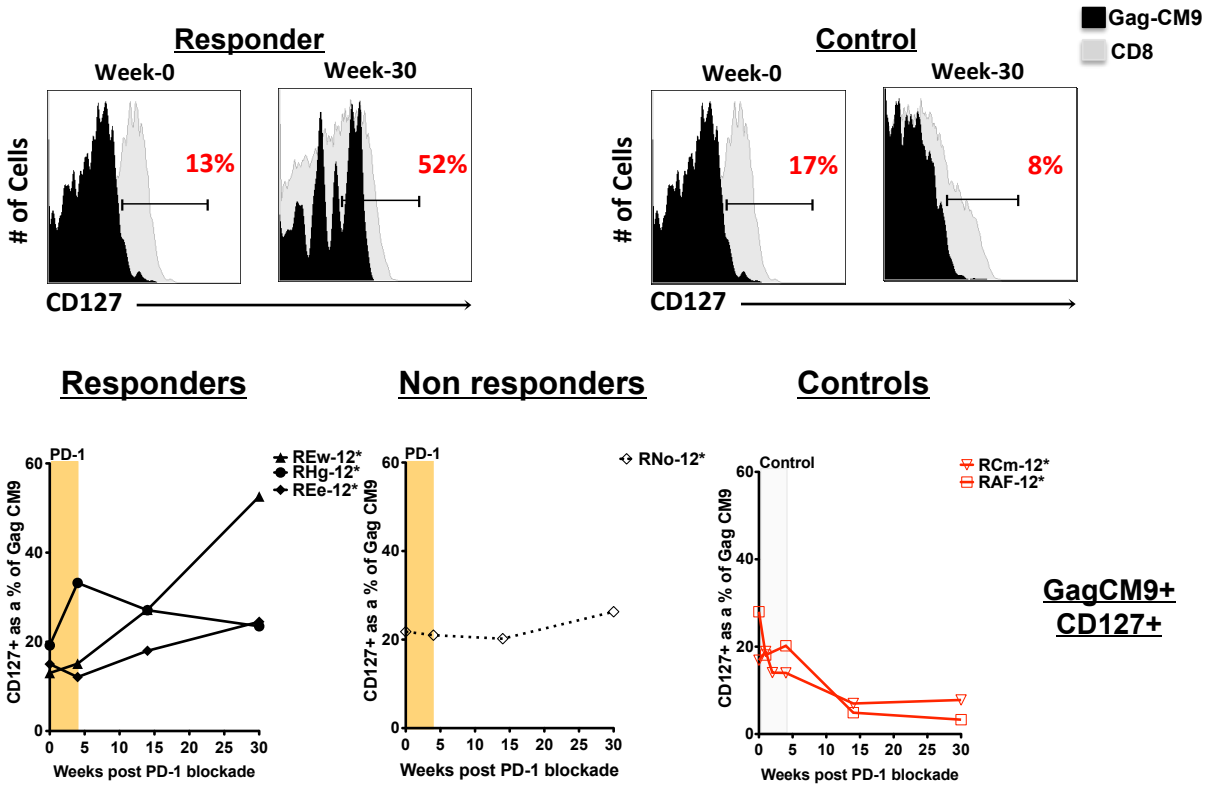

Fig 8. Temporal CD127+ Gag CM9-specific CD8 T cells in the blood of different group after PD-1 blockade post ART interruption. The frequency of CD127+ SIV-specific CD8 T cells increased in the responders.

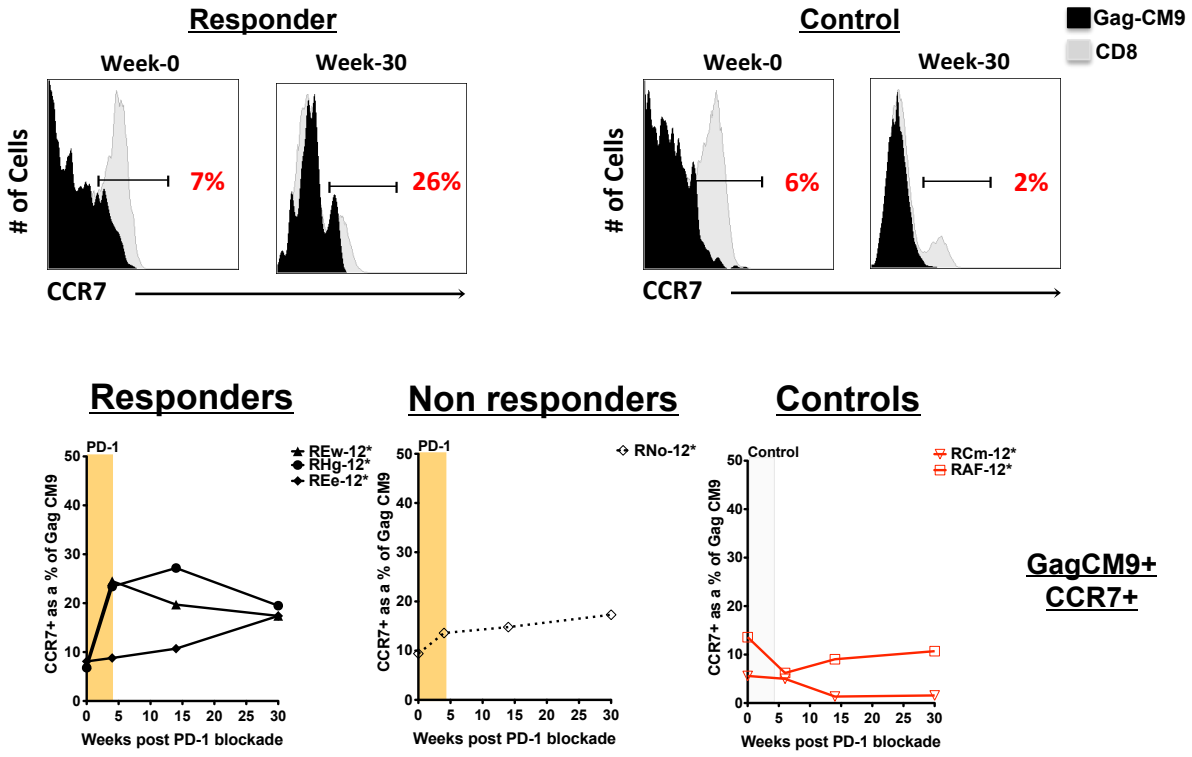

Fig. 9. Temporal CCR7+ Gag CM9-specific CD8 T cells in the blood of different groups after PD-1 blockade post ART interruption. The frequency of CCR7+ SIV-specific CD8 T cells increased in the responders.

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